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(54) Title: ION CHANNELS, IN PARTICULAR VANILLOID RECEPTOR – LIKE (VR–L) RECEPTOR

(57) Abstract

The invention provides isolated non–selective cation channel proteins which are (a) activatable by noxious heat; (b) obtainable from human T lymphocytes; (c) not capsaicin sensitive, particularly the VR–L protein (Seq ID No. 2), plus or variants (e.g. homologues or fragments) thereof. Also provided are nucleic acids encoding these (e.g. Seq ID No. 1), or which have utility in probing, amplifying or down–regulating these. Methods of providing the proteins and nucleic acids are disclosed, as are vectors, host cells, and organisms utilising them, and antibodies. Also disclosed are methods of influencing the electrophysiological and/or pharmacological properties of a cell (e.g. by way of therapy) based on manipulation of the proteins or nucleic acids, and methods for screening for substances having ion–channel modulating activity (which may be potential analgesics or other compounds which affect nociception, immunomodulatory agents or neuromodulatory agents).

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ION CHANNELS, IN PARTICULAR VANILLOID RECEPTOR – LIKE (VR-L) RECEPTOR

TECHNICAL FIELD

The present invention relates to ion channels, particularly those which are responsive, *inter alia*, to noxious heat, and nucleic acid encoding the same. It further relates to methods and materials for generating and using these.

PRIOR ART

A variety of proteinaceous ion channels have been characterised in the literature. One H^+-gated channel that is additionally activated by noxious heat and hot peppers is the 'VR1' channel (or 'vanilloid receptor-1') which was cloned from rat by Caterina et al. (1997) Nature, 389: 816-824. This may play a role in pain pathways.

Ion channels are of interest generally, *inter alia*, for their utility in investigating and manipulating the neurological and physiological processes which they mediate in *vivo*. In particular novel channels can be used to screen for agonists or antagonists which may exert desirable physiological effects (e.g. analgesics which affect nociception). Equally, proteins or nucleic acids based on the channels themselves may be used directly to manipulate these processes.

It will thus be appreciated that the provision of a new channel, particularly one having one or more novel activities compared to those already available to the public, would provide a contribution to the art.

DISCLOSURE OF THE INVENTION

The present inventors have cloned a novel ion channel from cultured Jurkat cells (a human leukemic T lymphocytes cell line). The protein functions as a non-selective ion channel

and has been shown to be heat-sensing.

The ion channel has structural similarities to VR1 sharing 47% identity therewith (based on the number of identical amino acids divided by the total number of amino acids in the region being compared).

The ion channel forming the basis of the present invention has been designated VR-Like or VR-L herein.

As with VR1, VR-L resembles members of the "trp" family of proteins in terms of topological organization Trp proteins are 6-transmembrane monomers first identified in *Drosophila* transient receptor potential (i.e. trp) mutants which shown deficits in photoreception. Both VR1 and VR-L have the characteristic N-terminal ankyrin repeats and considerable sequence similarity is also apparent in, but is limited to, the sixth transmembrane domain, its flanking sequences and the loop between transmembrane domains 5 and 6 believed to form part of the presumptive pore region. Certain trp genes provide the molecular basis for the phenomenon known as capacitative calcium entry which is loosely defined as the influx of Ca^{2+} from the extracellular space following inositol 1,4,5-triphosphate-induced mobilization of internal stores. It is known that trps play a critical role in phototransduction in *Drosophila*, and evidence that bradykinin, a hyperalgesic mediator, can gate trp-3 via activation of Gq heterotrimeric G-proteins has been obtained in a heterologous expression system. Studies by the Bargmann group in C. Elegans (Colbert et al. 1997, J Neurosci 17(21:8259-8269; Bargmann et al. 1998 Annu Rev Neurosci 21: 279-308) suggest that certain trp-related proteins (OSM-9) play a role in mechanosensory transduction as well.

VR1 itself is a capsaicin-gated channel. Such channels, present in sensory neurons, are believed to mediate the pain caused by acids and possibly other inflammatory mediators (e.g. bradykinin, prostaglandin E2 and 5-HT) which accompany tissue damage and ischaemic conditions. Heterologous expression of VR1 induces a capsaicin-sensitive cation channel which is transiently activated by rapid extracellular acidification or capsaicin. The biophysical and pharmacological properties of the VR1 channel closely match one of the capsaicin-gated cation channels described in sensory neurons (Helliwell et al, 1998 Neurosci Lett 10;250(3):177-180).

Like VR1, VR-L is expressed in sensory neurons and is likely to play a role in mediating the pain and inflammation accompanying tissue damage (nociception). Other endogenous ligands may also activate the receptor *in vivo*, either directly, or through G-protein coupled receptors by analogy with other TRP channels above. Thus this new channel is, *inter alia*, an analgesic or anti-inflammatory drug target.

However, unlike VR1, VR-L appears not to be capsaicin-sensitive under the conditions used in the experiments below. Additionally, it appears to have a different distribution to VR1 which, for instance, is not present in Jurkat cells. The structural and functional information about VR-L made available by the present inventors suggest that it may have additional utility to VR1.

Biro et al (1998) Blood 91: 1332-1340 reported that certain cells of the immune system (mast cell lines) take up calcium in response to capsaicin and RTX (a plant toxin which has similar actions to capsaicin). Biro et al. also showed, in

contrast to sensory neurons, capsaicin does not kill mast cells, or even induce their degranulation. However the putative receptor responsible for the demonstrated activity was not characterised.

The presence of VR-L in subsets of immune system cells suggests an important role as a receptor involved in the regulation of immune responses. Thus the channel also has utility in screening for agents that modulate immune responses.

Materials based on VR-L may be used in gene therapy both of disorders associated with sensory neurons (e.g. pain) and leucocytes (e.g. autoimmune disorders, leukemia).

The nucleic acid sequence and encoded protein of VR-L is shown within Fig 3A. The nucleic acid sequence is referred to as Seq ID No 1, while the encoded protein is Seq ID No 2.

Certain aspects of the present invention will now be discussed in more detail.

Thus in a first aspect of the present invention there is provided a non-selective cation channel protein which is activatable by noxious heat.

Channel proteins of the present invention may be obtainable from cells of the immune system, preferably human cells.

Preferably the channel proteins are not capsaicin sensitive under the conditions described in the Examples below. The channel may also be non-activatable by low pH i.e. not acid sensitive, by which is meant that the cation permeability of

the channel is not significantly increased by low pH (increased acidity, or reduced alkalinity) e.g. pH of 4-5, particularly pH 4.6.

Proteins, or polypeptides, of the present invention may be provided in recombinantly produced, isolated, enriched or cell-free form. They may be present in cells heterologously, which is to say that they do not naturally occur there, but have been introduced through human intervention.

By "non-selective" is meant that the activated channel is permeable to more than one cation, preferably at least Na^+, K^+, Ca^{2+}, preferably having a broadly comparable permeability to each.

It is activatable by noxious heat i.e. heat at a level which may typically stimulate a pain response e.g. greater than around 45°C, 50°C or 55°C or higher.

The various activities of the channel can be assessed by those skilled in the art without undue burden, for instance using voltage clamping techniques analogous to those described herein to analyse membranes or membrane preparations (e.g. from eucaryotic cells) into which the protein has been expressed or otherwise introduced. Some generally applicable methods are discussed in "Ionic Channels of Excitable Membranes" Hille, B. - Pub 1992, Sinauer.

Preferably the protein will have one or more of the electrophysiological and pharmacological characteristics of the VR-L protein described herein. Preferably the protein comprises amino acid sequence Seq ID No 2.

In a further aspect of the invention there is disclosed a VR-L variant protein having ion channel activity and comprising an amino acid sequence having at least about 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% sequence identity with Seq ID No 2. As noted above, the VR1 channel of the prior art shares about 47% identity (56% similarity) with Seq ID No 2.

Ion channel activity may be tested as described above, using appropriate stimuli if required.

Similarity (or identity, or homology) may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman.

Parameters are preferably set, using the default matrix, as follows:

Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA
Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA
KTUP word length: 2 for proteins / 6 for DNA.

Homology may be over the full-length of the relevant sequence shown herein, or may be over a part of it, preferably over a

contiguous sequence of about or greater than about 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 300, 400, 500, 600, 700 or more amino acids or compared with Seq ID No 2.

Thus a variant polypeptide in accordance with the present invention may include within the sequence shown in Seq ID No 2, a single amino acid or 2, 3, 4, 5, 6, 7, 8, or 9 changes, about 10, 15, 20, 30, 40 or 50 changes, or greater than about 50, 60, 70, 80 or 90 changes. In addition to one or more changes within the amino acid sequence shown, a variant polypeptide may include additional amino acids at the C-terminus and/or N-terminus. Alternatively it may represent an active (as an ion channel) fragment of the protein e.g. a pore forming fragment.

Proteins or polypeptides of the present invention may be prepared by the expression of nucleic acids encoding therefor in appropriate host cells, as described in more detail hereinafter.

In a further aspect of the present invention there is provided nucleic encoding a protein of the invention as described above.

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs (e.g. peptide nucleic acid). Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

Nucleic acid molecules according to the present invention may be provided isolated and/or purified from their natural

environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities.

The nucleic acid molecules may be wholly or partially synthetic. In particular they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially. Alternatively they may have been synthesised directly e.g. using an automated synthesiser.

Thus in one embodiment of this aspect of the invention there is provided a nucleic acid encoding VR-L e.g. a nucleic acid encoding Seq ID No 2 (which nucleic acid sequence may be that set out within Seq ID No 1). In another embodiment there is disclosed a sequence degeneratively equivalent to that sequence.

In another embodiment there is disclosed a nucleic acid which encodes a VR-L variant as described above. Generally this will share homology or identity with the VR-L encoding sequence in similar terms as are described in relation to the variant proteins above, but wherein amino changes correspond to changes in codons or individual nucleotides.

Variants may include distinctive parts or fragment (however produced) corresponding to a portion of the sequence provided. The fragments may encode particular functional parts of the polypeptide.

Equally the fragments may have utility in probing for, or amplifying, the sequence provided or closely related ones.

Suitable lengths of fragment, and conditions, for such processes are discussed in more detail below.

Also included are nucleic acids which have been extended at the 3' or 5' terminus with respect any of these embodiments.

Preferred variant nucleic acids are those which, in addition to encoding VR-L variants, are capable of hybridizing with a poly- or oligonucleotide having a sequence which is complementary to a distinctive portion of SEQ ID No 1 under low stringency conditions, more preferably being capable of hybridizing with one or more of such poly- or oligonucleotides under high stringency conditions.

The expressions 'low stringency conditions' and 'high stringency conditions' will be understood by those skilled in the art, but are conveniently exemplified as set out in US 5202257, Col 9-Col 10. For instance, screening may initially be carried out under conditions, which comprise a temperature of about 37°C or less, a formamide concentration of less than about 50%, and a moderate to low salt (e.g. Standard Saline Citrate ('SSC') = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7) concentration.

Alternatively, a temperature of about 50°C or less and a high salt (e.g. 'SSPE'= 0.180 mM sodium chloride; 9 mM disodium hydrogen phosphate; 9 mM sodium dihydrogen phosphate; 1 mM sodium EDTA; pH 7.4). Preferably the screening is carried out at about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5 X SSC, or a temperature of about 50°C and a salt concentration of about 2 X SSPE. These conditions will allow the identification of sequences which have a substantial degree of homology (similarity, identity)

with the probe sequence, without requiring the perfect homology for the identification of a stable hybrid.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): T_m = 81.5°C + 16.6Log [Na+] + 0.41 (% G+C) - 0.63 (% formamide) - 600/#bp in duplex

Suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

Sequence variants which occur naturally may include alleles (which will include polymorphisms or mutations at one or more bases) or pseudoalleles. Also included within the scope of the present invention would be isogenes, or other homologous genes from other species sharing similarity with SEQ ID No 1.

The term 'variant' nucleic acid as used herein encompasses all of these possibilities.

Thus in a further aspect of the present invention there is provided a method of identifying and/or cloning a nucleic acid according to the present invention (i.e. encoding an ion channel as described above) which method employs Seq ID No 1 or a distinctive fragment or region thereof.

By 'distinctive' is meant based on a region which is not present in VR1 or other trp proteins. The sequence information in the region can be used directly to identify or clone variants, or be used to identify corresponding regions in a data-base (e.g. of expressed sequence tags, or sequence tagged sites), optionally from a different species.

For instance, using the information disclosed herein, VR-L homologues in both rat and mouse have been identified. Portions of the encoded amino acid sequences are shown in Fig 4.

Thus in a further embodiment, a variant in accordance with the present invention is also obtainable by means of a method which includes:
(a) providing a preparation of nucleic acid, e.g. from cells of the immune system, or from sensory neurons,
(b) providing a nucleic acid molecule having a nucleotide sequence shown in or complementary to Seq ID No 1 or a distinctive fragment thereof,
(c) contacting nucleic acid in said preparation with said nucleic acid molecule under conditions for hybridisation of said nucleic acid molecule to any said gene or homologue in said preparation, and identifying said gene or homologue if present by its hybridisation with said nucleic acid molecule.

Probing may optionally be done by means of so-called 'nucleic acid chips' (see Marshall & Hodgson (1998) Nature Biotechnology 16: 27-31, for a review).

Nucleic acids of the invention may be amplified from template DNA from cells using a specific DNA amplification reaction with specific primers targeted to amplify the DNA required,

e.g. of SEQ ID No 1, e.g. from genomic DNA, DRG cDNA, or mRNA templates, e.g. by using polymerase chain reaction or, from RNA, by using reverse transcription (RT) followed by polymerase chain reaction (PCR)(see "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, 1990).

Thus a method involving use of PCR in obtaining nucleic acid according to the present invention may include:
(a) providing a preparation of nucleic acid, e.g. from DRG, or other appropriate tissue or organ,
(b) providing a pair of nucleic acid molecule primers useful in (i.e. suitable for) PCR, at least one said primers having a sequence shown in or complementary to a sequence shown in Seq ID No 1 or a distinctive fragment thereof,
(c) contacting nucleic acid in said preparation with said primers under conditions for performance of PCR,
(d) performing PCR and determining the presence or absence of an amplified PCR product. The presence of an amplified PCR product may indicate identification of a variant.

In all cases, if need be, clones or fragments identified in the search can be extended. For instance if it is suspected that they are incomplete, the original DNA source (e.g. a clone library, mRNA preparation etc.) can be revisited to isolate missing portions e.g. using sequences, probes or primers based on that portion which has already been obtained to identify other clones containing overlapping sequence.

Distinctive fragments or oligonucleotides for use in probing or PCR may be selected prepared by those skilled in the art without burden in the light of the present disclosure. Typically they may be about 10 to 30 or fewer nucleotides in

length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16-24 nucleotides in length may be preferred. If required, probing can be done with entire restriction fragments of the gene disclosed herein which may be 100's (e.g 500 or more) or even 1000's of nucleotides in length.

Such oligonucleotides, and their complements (but excluding any oligonucleotides consisting solely of regions disclosed *per se* in the form of EST clones, or other portions of undefined function) form one part of the present invention.

Artificial variants (derivatives) may be prepared by those skilled in the art on the basis of the sequences provided herein, for instance by site directed or random mutagenesis, or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly (e.g. via one or more amplification or replication steps) from an original nucleic acid having all or part of the sequence shown in Seq ID No 1.

Thus in a further aspect of the invention there is disclosed a method of producing a derivative nucleic acid comprising the step of modifying the coding sequence of Seq ID No 1.

Changes to a sequence, to produce a derivative, may be by way of one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide.

Changes may be desirable for a number of reasons, including introducing or removing the following features: restriction

endonuclease sequences; codon usage; other sites which are required for post translation modification; cleavage sites in the encoded polypeptide; motifs in the encoded polypeptide for post-translational modification. Leader or other targeting sequences (e.g. membrane or golgi locating sequences) may be added to the expressed protein to determine its location following expression. All of these may assist in efficiently cloning and expressing an active polypeptide in recombinant form.

Other desirable mutation may be random (e.g. chemical) or site directed mutagenesis in order to alter the activity (e.g. specificity) or stability of the encoded polypeptide. For such methods, for example, a vectorised DNA of SEQ ID No 1 is exposed to a mutagenic material such as hydroxylamine, or, in the case of SDM, a PCR reaction is carried out on that DNA using a mutagenic primer, whereby DNA is produced which encodes for a protein different in sequence to SEQ ID No 2 at a few predictable or predetermined sites respectively.

Particular target areas of the sequence are those which may determine any of the following: specificity of activation of the ion channel (e.g. whether or not a given stimulus, such as pH, elicits a response) or specificity of the ion channel (e.g. relative permeability to different cations).

All of these properties can be tested, without burden, e.g. using appropriate voltage clamping techniques.

Preferably the amino acid sequence differs from SEQ ID No 2 only by conservative substitutions. The expression 'conservative substitutions' as used with respect to amino acids relates to the substitution of a given amino acid by an

amino acid having physicochemical characteristics in the same class. Thus where an amino acid in SEQ ID No 2 has a hydrophobic characterising group, a conservative substitution replaces it by another amino acid also having a hydrophobic characterising group; other such classes are those where the characterising group is hydrophilic, cationic, anionic or contains a thiol or thioether. Such substitutions are well known to those of ordinary skill in the art (see e.g. US 5380712) and are only contemplated where the resultant protein has ion channel activity.

Also included are variants having non-conservative substitutions. As is well known to those skilled in the art, substitutions to regions of a peptide which are not critical in determining its conformation may not greatly affect its activity because they do not greatly alter the peptide's three dimensional structure. In regions which are critical in determining the peptides conformation or activity such changes may confer advantageous properties on the polypeptide.

In one aspect of the present invention, the nucleic acids of the present invention are in the form of a recombinant and preferably replicable constructs, such as vectors.

"Vector" is defined to include, inter alia, any plasmid, cosmid, phage or other vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Specifically included are shuttle vectors by which is meant a

DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eucaryotic (e.g. mammalian, yeast or fungal cells).

A vector including nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic VR-L DNA (isolatable as described above) this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter operatively linked to a nucleotide sequence provided by the present invention, such as the VR-L gene (see Seq ID NO 1) or a variant thereof.

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press.

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis (see above discussion in respect of variants), sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

In one embodiment of this aspect of the present invention provides a gene construct, preferably a replicable vector, comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention, such as Seq ID No 1.

The term "inducible" as applied to a promoter is well

understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus.

Particularly preferred are vectors suitable for expression of mammalian DNA, such as will occur to those skilled in the art, e.g. HSV or vaccinia vectors, or pcDNA3 shuttle vectors, e.g. as included within the lambda express system (Stratagene), which are capable of expressing heterologous protein in both bacteria and in eucaryotic cells such as COS cells. Suitable bacterial vectors will include lambda-Zap vectors such as the lambda-Zap-II vector available from Stratagene Cloning Systems. Bacterial clones containing plasmids capable of gene expression can be obtained by excising pBluescript from the lambda-Zap-II construct in the presence of a filamentous helper phage also available from Stratagene. Typical protocols are provided in the examples below, in Stratagene kit inserts.

A further aspect of the present invention provides cells containing, or more preferably transformed with (or transfected with) the nucleic acids of the present invention. Such cells are provided by transformation of a host cell, preferably a eucaryotic cell, e.g. a COS, CHO or HEK 293 cell or an oocyte, preferably a Xenopus oocyte, particularly COS cells, using DNA of the invention as incorporated by

recombinant DNA techniques into a vector or as directly incorporated into the cells' genomic DNA e.g. by electroporation or other such DNA integrating technique.

Such cells may be capable of expressing, or having expressed, a VR-L protein as described above. The cells may thus mimic, in some respects, the electrophysiological and pharmacological properties of native VR-L-expressing cells

It is also possible to produce cells bearing the receptor protein of the invention by direct injection of RNA of the present invention into the cells wherein it becomes translated.

The foregoing discussion has been generally concerned with uses of the nucleic acids of the present invention for production of functional polypeptides, thereby increasing the VR-L or related ion channel activity in the cell.

However the information disclosed herein may also be used to reduce the activity of VR-L or variants thereof in cells in which it is desired to do so.

For instance down-regulation of expression of a target gene may be achieved using anti-sense technology.

In using anti-sense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. See, for example, Rothstein et al, 1987; Smith *et al*,(1988) *Nature* 334, 724-726; Zhang *et al*,(1992) *The Plant*

Cell 4, 1575-1588, English *et al.*, (1996) *The Plant Cell* 8, 179-188. Antisense technology is also reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

"Complementary to" means capable of base pairing with, whereby A base pairs with T (and U); G base pairs with C.

The "complement" of a reference nucleic acid consists of a sequence of nucleotides which are the counterpart of the entire nucleotide sequence of that reference nucleic acid.

An alternative to anti-sense is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in expression of the target gene by co-suppression.

Further options for down regulation of gene expression include the use of ribozymes, e.g. hammerhead ribozymes, which can catalyse the site-specific cleavage of RNA, such as mRNA (see e.g. Jaeger (1997) "The new world of ribozymes" Curr Opin Struct Biol 7:324-335, or Gibson & Shillitoe (1997) "Ribozymes: their functions and strategies form their use" Mol Biotechnol 7: 242-251.)

Methods for producing regulatory sequences will involve no burden to those skilled in the art in the light of the present disclosure. Anti-sense or sense or ribozyme based regulation may be performed using vectors as described above, and may itself be regulated by employing an inducible promoter in an appropriate construct. For instance incorporation of this DNA into mammalian cells might be readily accomplished using vectors, e.g. such as HSV, vaccinia or adenovirus (see

Principles of Gene Manipulation (1994) 5th Edit. Old and Primrose 5th Edition, Blackwell Scientific Publications).

The complete sequence corresponding to the coding sequence (in reverse orientation for anti-sense) need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is characteristic of one or more genes, such as a regulatory sequence.

The sequence employed may be about 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides, about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than about 500 nucleotides are preferable where possible, such as longer than about 600 nucleotides, than about 700 nucleotides, than about 800 nucleotides, than about 1000 nucleotides or more.

It may be preferable that there is complete sequence identity in the sequence used for down-regulation of expression of a target sequence, and the target sequence, although total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the sequence used from the target gene. Thus, a sequence employed in a down-regulation of gene expression in accordance with the present

invention may be a wild-type sequence (e.g. gene) selected from those available, or a variant of such a sequence.

The sequence need not include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is about 5%, 10%, 15% or 20% or more mismatch between the sequence used and the target gene. Effectively, the homology should be sufficient for the down-regulation of gene expression to take place.

One embodiment of this aspect of the invention employs DNA oligonucleotides, typically being of 10 to 30 bases long, conveniently about 20 bases long, optionally in degradation protected form, e.g. by being thiolated, and which conveniently have been chemically synthesized to be directed to hybridize with a part of the 5' coding region of the VR-L mRNA. Annealing with the oligomeric DNA causes the mRNA to be degraded by activation of RNase H, or blocks the translation of the mRNA into protein. The small size of such oligomers facilitates their direct access into target cells which express the present VR-L proteins.

An alternative embodiment produces antisense RNA in vivo by inserting a tissue specific inducible or constitutively active promoter, enhancer or locus control region or element upstream of the coding region, or part of the coding region, of DNA of SEQ ID No 1 in a construct which is then cloned into a vector. For use in mammals in therapy such a vector should be capable of infecting but not killing target cells. Convenient vectors for use in this embodiment, which can target mammalian dorsal

root ganglion cells are Herpes Simplex Virus (HSV) vaccinia or adenovirus derived vectors. Viral vectors for use in gene therapy are discussed by Vile (1997) Nature Biotechnology 15: 840-841. A non-viral gene therapy approach is discussed by Sebestyen et al (1998) Nature Biotechnology 16: 80-85. The use of a variety of gene therapy delivery systems (including HSV VP22) is discussed by Fernandez & Baylay (1998) Nature Biotechnology 16: 418-420 and references therein.

Where the antisense downregulating DNA or RNA is provided in dorsal root ganglia cells it potentially inhibits the pain response by actually decreasing the number of VR-L channels on the surface of sensory cells. Where the antisense downregulating DNA or RNA is provided in the immune system, it alters leucocyte function by actually decreasing the number of VR-L channels on the surface of neurons.

Thus a nucleotide sequence which is complementary to any of the coding-nucleic acids discussed in relation to earlier aspects of the invention forms one part of the present invention.

The invention further provides a method of influencing the electrophysiological and pharmacological properties of a cell, said method comprising the step of causing or allowing expression of a heterologous nucleic acid sequence as discussed above within the cell.

The present invention further provides the use of the nucleotide sequence of Seq ID No 1, or its complement, or a variant of either for down-regulation of gene expression, particularly down-regulation of expression of an ion channel-encoding gene, more preferably a cation channel, most

preferably VR-L.

In a further aspect of the present invention there is provided nucleic acid (e.g. antisense DNA) of the present invention for use gene therapy, or for use in the preparation of medicaments for use in gene therapy.

In a further aspect of the present invention there is disclosed an organism, preferably a non-human mammal, comprising cells in which the activity of VR-L or a variant thereof have been altered, preferably impaired, by use of the methods and materials discussed above. Particularly preferred is a rodent e.g. murine organism. Methods of producing 'knock out' mammals in which specific gene activities have been impaired are now well known to those skilled in the art - see e.g. Boerrigter et al (1995) Nature 377: 657-659, or Gossen and Vijk (1993) Trends Genet 9: 27-31.

In a further aspect of the present invention there is provided use of a protein, a transformed or transfected cell, or a transgenic organism as described above, for identifying a substance as having ion-channel modulating activity.

Such substances may, for instance, act as agonist, partial agonist or antagonist.

Thus the protein or cell may be used in a method comprising exposing the protein (e.g which is associated with a membrane, for instance of a liposome) or cell surface to a solution of the substance such as to allow interaction between the substance and the VR-L (or variant VR-L) protein in the membrane and then measuring the electrophysiological response of the cell or membrane to this interaction. This measurement

may optionally be compared with a reference figure.

Typically the response may be measured by use of a microelectrode technique accompanied by such measurement strategies as voltage clamping of the cell whereby activation of ion channels may be identified by inward or outward current flow as detected using the microelectrodes. ^{22}Na, ^{86}Rb, ^{45}Ca radiolabeled cations or ^{14}C or ^{3}H guanidine may be used to assess such ion flux; a sodium, calcium or potassium ion sensitive dye (such as Fura-2, or indo) may be used to monitor ion passage through the receptor ion channel, or a potential sensitive dye may be used to monitor potential changes, e.g.. such as in depolarization.

Agonists and partial agonists may be identified by their relative efficacy as compared with other known agonists and stimuli in activating the receptor or, in the case of partial agonists and antagonists, by their ability to block the activation caused by other known agonists.

Such substances will have potential as analgesics or other immuno- or neuro-modulatory agents, and as such form a further aspect of the present invention, optionally in the form medicamanents (e.g. compositions comprising a pharmaceutically acceptable carrier or filler).

Purified VR-L protein, or a variant thereof, e.g. produced recombinantly by expression from encoding nucleic acid therefor, may be used to raise antibodies employing techniques which are standard in the art.

Antibodies and other polypeptides comprising antigen-binding fragments of antibodies may be used (*inter alia*) as

antagonists or inhibitors, or for use in identifying homologues.

Methods of producing antibodies include immunising a mammal with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest.

For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82). Antibodies may be polyclonal or monoclonal.

Antibodies may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any specific binding substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of Chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023. It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the Vl and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii)

single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

The present invention will now be described by way of illustration only by reference to the following non-limiting Figures and Examples. Further embodiments of the invention falling within the scope of the claims provided herewith will occur to those of ordinary skill in the art in the light of these.

FIGURES

Fig 1: 'virtual transcript' of VR-L based on overlapping human EST clones. Analogous positions in VR1 from where primers were designed are indicated by numbers above the map. Primers and their orientations are indicated by arrowheads. Dashed vertical lines flank gaps not represented by any EST sequence. The 5' untranslated region (UTR) and the regions surrounding the stop codon were deduced from results of tentative human consensus sequence queries of the human genome index.

Fig 2: generation of full length sequence from PCR products by trans-PCR (Fig 2A) or utilisation of unique restriction site (Fig 2B).

Fig 3A: VR-L nucleotide and amino acid sequences.

Annotations: Ankyrin like repeats (ANK), predicted transmembrane helices (TM) and the possible pore loop are designated with (*), (_) and (^), respectively.

Fig 3B: amino acid sequence alignment of rat VR1 (upper) and human VR-L (lower).

Fig 4: multiple sequence alignment of rat VR1 with mouse, rat and human (Jurkat cell) VR-L in the transmembrane 5/6 region.

Fig 5: whole cell voltage clamp recordings of COS-7 cells transfected with VR-L.

Fig 6: Table 1; EST clones showing homology to rat vanilloid receptor subtype 1 (VR1)

Fig 7: Table 2; primers used for cloning VR-L fragments.

EXAMPLES

Example 1- Cloning of VR-2

Overview

An innovative "virtual cloning" approach to finding VR-L was adopted. A tblastn search of the Genbank dbEST identified at least 30 human and mouse entries (Fig 6, Table 1) showing considerable homology to different regions of the rat VR1 amino acid sequence. The overlapping human EST clones were assembled into a virtual transcript (Fig. 1), albeit interrupted by gaps. The contigs formed represent sequences which were broadly analogous to positions 86 to 763 of the rat VR1 amino acid sequence.

Since the EST clones were obtained from different cDNA libraries, and hence the contigs may comprise ESTs derived from different tissues, there was no way of knowing whether or not the 'transcript' was real. An RT-PCR-based approach involving overlapping primer sets (see Fig. 1 and Fig 7, Table 2) was therefore used to fill in the gaps between the different contigs and to establish whether or not these clones did indeed form part of a single transcript. Total RNA derived from Jurkat cells (a leukemic T cell line; and from where an EST clone was derived) was used as a template for reverse transcription. Sequencing of PCR products confirmed that the individual entries were one and the same transcript truncated at various points during reverse transcription.

As shown in Fig 1, the tblastn search of the EST databases did not pull out any clone with apparent homology to the amino terminal region of VR1.

A search of the Human Gene Index (HGI) THC database of The Institute for Genome Research (TIGR) was made using as query the EST clones homologous to the more 5' region of VR1 (GenBank accession nos. H20025, AA461295, H51393, AA236417) revealed more 5' sequences (THC 176254) contiguous with the other EST clones but divergent from those of VR1. The putative initiation methionine was assigned based on identification of a stop codon in what should be the 5' untranslated region of the transcript.

The human EST clone with virtually identical amino acid sequences to the 3'-most region of VR1 (GenBank accession no. AA321554) turned out not to be part of the transcript formed by all the other EST(s) pulled out, implying that there are indeed at least two vanilloid receptor subtypes

(and that AA321554 may form part of a human VR-1 homologue). Primers based on this EST, in combination with primers from the more 5' region of VR-L (see Figure 1), resulted in failed PCR(s) or did not give a distinct PCR product with any similarity to either VR1 or VR-L.

A subsequent THC database search using the more 3' VR-L EST clones as query (GenBank accession nos. AA281348, W92895, N35179, T12251) pulled out more 3' sequences (THC161190) which led to the identification of the real 3' end bearing both stop codon and 3' untranslated sequences (which are quite divergent from those of VR1) . Primers designed from this putative 3' end, in combination with more 5' forward primers, gave distinct products which were later verified by sequencing to be contiguous with the rest of the VR-L sequences. The longer PCR products were spliced together both by trans-PCR (splicing by overlap extension) or by ligation utilizing a unique restriction site in the area of overlap (Figs.2a and 2b). Fig. 3b shows a comparison of the human VR-L amino acid sequence with that of rat VR1.

To further demonstrate that these clones do not simply represent the human homologue of rat VR1, VR-L-specific PCR primers based on a mouse EST clone (GenBank accession no. AA476107) were used to amplify VR-L from rat dorsal root ganglia cDNA from which VR1 was isolated. Sequencing revealed a transcript similar to but distinct from VR1, confirming that there are at least two vanilloid receptor subtypes in the rat genome. An alignment of the rat VR1 with the rat, mouse and human VR-L sequences in this region is shown in Fig. 4.

RT-PCR and Subcloning Procedures

Total RNA was extracted from cultured Jurkat cells (leukemic T lymphocytes) following the method of Chomcyznski and Sacchi (Analytical Biochemistry 162: 156-159, 1987). Reverse transcription was carried out as follows. Total RNA (2 μg) and 50 ng of primer (random hexamers or gene-specific oligonucleotides), in a total volume of 9 μl, was heated to 70°C for 10 min, to disrupt possible secondary structures. The mixture was then incubated at 37°C for 2 min in 1 x buffer [50mM Tris-HCl (pH 8.3), 75 mM KCl, 3mM $MgCl_2$], 10 mM dithiothreitol and 125 mM of each dNTP (dATP, dCTP, dGTP and dTTP). Superscript II reverse transcriptase (200 U) was added to a final volume of 20 μl and the mixture was incubated for 60 minutes at 37°C. The reaction was stopped by a 5-minute incubation at 94°C.

For the initial PCR amplifications, reactions were carried out using 1 μl of first strand cDNA, 40 pmol of each primer, 1x buffer [50 mM KCl, 10mM Tris-HCl pH9.0, 0.1% Triton X-100], 1.5 mM MgCl2, 0.2mM dNTPs and 1U of Taq polymerase in a final volume of 20 μl. The amplification program used included 1 cycle of 94°C for 5 min, 53-57°C for 1 min, 72°C for 1-2 min; 35 cycles of 94°C for 1 min, 53-57°C for 45 sec, 72°C for 1-2 min; and a final extension at 72°C for 5 min. For cloning purposes, 0.4 U of Gibco-BRL's eLONGase enzyme mix (a mixture of Taq polymerase and the proofreading Pyrococcus species GB-D thermostable DNA polymerases) was used in a 20 μl reaction including 1 μl first strand cDNA, 40 pmol of each primer, 1x buffer [60 mM Tris-SO_4 (pH9.1), 18 mM $(NH_4)_2SO_4$ with $MgSO_4$ between 1.5 to 1.9 mM] and 0.2 mM

dNTPs. Cycling conditions were: 1 cycle of 94°C for 3 min, 55-57°C for 1 min, 68°C for 45 sec to 2 mini 30-35 cycles of 94°C for 30 sec, 55-57°C for 30 sec, 68°C for 45 sec to 2 min; and a final extension at 68°C for 5 minutes. PCR products were cloned into the vector pGem-T Easy (Promega) and sequenced using standard procedures. For blunt-ended PCR products generated by eLONGase, a 10 minute incubation with 1U Taq polymerase after the PCR reaction was necessary to add an "A" overhang necessary for TA-cloning into the pGem-T Easy vector.

The larger PCR products were spliced together to obtain the full-length VR-L clone, either by trans-PCR (splicing by overlap extension) or conventional ligation involving a unique restriction site (see Figs. 2a and 2b). The full-length clone was then transferred into a mammalian expression vector (pGW-1) and checked for correct orientation. The resulting recombinant clone was subsequently used for transfection of COS-7 cells by electroporation.

Example 2 - VR-L Expression in COS-7 Cells.

The full length VR-L clone (in pGemT easy) was cut out with EcoRI and subcloned into the EcoRI site of pGW1. The orientation of the resulting construct was verified by restriction analysis and sequencing (see Fig 2A).

Shuttle vectors e.g. pTracer-CMV or pgw1, containing VR-L were propagated and purified from bacterial cultures transformed with the recombinant plasmid. These vectors were used to express ion channels by transfecting COS cells. Cultured COS cells from a 100 mm petri dish (80-

90% confluent) were trypsinised and resuspended in 350 microlitres of ice cold HEBS buffer. 20-30 micrograms of plasmids of interest were dissolved with 150 microlitres of HEBS buffer, then mixed with the COS cell suspension in an electroporation cuvette and kept on ice to cool for 5 minutes. Meanwhile, the electroporator (Invitrogen) was set up at 250 microFarad, and charged for 3 minutes at 330V, 25mA, and 25W. The cuvette was flicked to resuspend cells and electroporation effected.

After transfection by electroporation, COS cells were seeded in low density onto 35mm petri dishes and cultured with 2ml MEM/10% FCS at 37°C for 1-2 days.

Example 3 - VR-L electrophysiology

Whole-cell voltage-clamp recordings were made 1-2 days after transfection.

Membrane currents were recorded using an Axopatch 200B amplifier. Currents were low-pass filtered at 5kHz (4-pole Bessel filter), and digitized using a Digidata 1200 interface. Acquisition and analysis of currents was achieved using pClamp6 software. Pipettes were filled with an intracellular solution containing (in mM); KCl 120, NaCl 8, MgCl2 3, HEPES 40, and BAPTA 10, at pH 7.35. Recordings were made at room temperature (18-22°C).

The extracellular recording solution was composed of (in mM); NaCl 146; KCl 5; Glucose 10; $MgCl_2$ 1, $CaCl_2$ 0.01. For extracellular solutions with pH values of 7.4-6.5, 10mM HEPES was used as the buffer, whilst for solutions of pH 6.5-4.0, MES at a concentration of 10mM was used.

Results

VR-L forms a functional channel when transiently expressed in COS-7 cells. Despite the homology of VR-L to VR1, the channel does not appear to be gated by capsaicin or low pH.

However, exposure of VR-L-expressing COS-7 cells to noxious heat does evoke inward currents, making this channel significantly different in its function in comparison to VR1.

Figure 5 shows responses from a typical cell. Application of 10 μM capsaicin to this cell, at a holding potential of -60mV, failed to evoke a change in membrane current (1A) Bath application of extracellular recording solution at room temperature caused an increase in current noise (1B), presumably due to mechanical disturbance. A similar application of extracellular solution at 55°C evoked a large inward current (1C). These studies suggest that the channel is non-selective with respect to cation permeability. Thus it would appear that native cells expressing the channel would respond to conditions of noxious heat. Since the current would be inward at resting membrane potentials the overall effect would be one of excitation. On activation of the channel, depolarisation would occur causing voltage-gated sodium and calcium channels to be recruited, further enhancing the excitatory effect.

Example 4: *Screening for ion channel modulating agents*

Compounds to be assessed as agonists, partial agonists or

antagonists of the VR-L channels may be bath applied in the system above and inward current used as measurement of the activation or block of channels encoded by the transfected vectors.

Alternatively, permanently or transiently transfected cell lines expressing VR-L are used to measure increases in intracellular calcium in response to the stimuli discussed in Example 3 with or without the addition of compounds that may show agonist, partial agonist or antagonist activity.

Changes in intracellular calcium are measured using cells in multiwell plates using Ca-45 uptake, for instance using the method of Wood et al (1989) J Neurochem 53: 1203-1211, or activation of calcium sensitive dyes (e.g. Fura-II) as described in Zeilhofer et al (1996) J Neurophysiol 76(5): 2834-40 or Hansen et al (1998) Am J Physiol 274(6 Pt 1): C1552-62.

Claims

1 An isolated non-selective cation channel protein which is:
(a) activatable by noxious heat,
(b) obtainable from human T lymphocytes,
(c) not capsaicin sensitive.

2 An isolated VR-L protein comprising amino acid sequence Seq ID No 2.

3 An isolated VR-L variant protein which:
(a) has ion channel activity, and
(b) comprises an amino acid sequence having at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with Seq ID No 2.

4 An isolated nucleic acid comprising a nucleotide sequence encoding a protein as claimed in any one of the preceding claims.

5 A nucleic acid as claimed in claim 4 wherein the nucleotide sequence comprises Seq ID No 1 or a sequence degeneratively equivalent thereto.

6 A nucleic acid as claimed in claim 4 wherein the nucleotide sequence encodes the VR-L variant of claim 3.

7 A nucleic acid as claimed in claim 4 which is capable of hybridising with a nucleic acid comprising a sequence which is complementary to Seq ID No 1.

8 A nucleic acid as claimed in claim 6 or claim 7 wherein

the nucleotide sequence encodes an allelic variant of Seq ID No 1.

9 A nucleic acid as claimed in claim 6 or claim 7 wherein the nucleotide sequence encodes is a VR-L variant from of non-human origin.

10 A nucleic acid as claimed in claim 9 wherein the nucleotide sequence encodes an amino acid sequence of Fig 4.

11 A nucleic acid as claimed in claim 6 or claim 7 wherein the nucleotide sequence is a derivative of Seq ID No 1 by way of one or more of addition, insertion, deletion or substitution of one or more nucleotides therein.

12 A nucleic acid which is the complement of the nucleic acid of any one of claims 4 to 11.

13 A nucleic acid molecule for use as a probe consisting of a distinctive nucleotide sequence of at least 30 contiguous nucleotides of Seq ID No 1, or a sequence degeneratively equivalent thereto, or complementary to either.

14 A nucleic acid molecule for use as a primer consisting of a distinctive nucleotide sequence of between 10 and 30 contiguous nucleotides of Seq ID No 1, or a sequence degeneratively equivalent thereto, or complementary to either.

15 A method of identifying and/or cloning a nucleic acid according to any one of claims 4 to 12 which method employs a probe or primer of claim 13 or claim 14.

16 A method as claimed in claim 15, which method includes the steps of:
(a) providing a preparation of nucleic acid,
(b) providing a probe as claimed in claim 13,
(c) contacting nucleic acid in said preparation with said probe under conditions for hybridisation, and
(d) identifying any nucleic acids which hybridise with said probe.

17 A method as claimed in claim 15, which method includes the steps of:
(a) providing a preparation of nucleic acid,
(b) providing a pair of nucleic acid molecule primers suitable for PCR, at least one of said primers being a primer as claimed in claim 14,
(c) contacting nucleic acid in said preparation with said primers under conditions for performance of PCR,
(d) performing PCR and determining the presence or absence of an amplified PCR product.

18 A method of producing a derivative nucleic acid comprising the step of modifying the nucleic acid of claim 5.

19 A method as claimed in claim wherein ion channel protein encoded by the derivative nucleic acid is modified with respect to its specificity of activation or permeability.

20 A recombinant vector comprising the nucleic acid of any one of claims 4 to 12.

21 A vector as claimed in claim 20 wherein the nucleic acid is operably linked to a promoter or other regulatory element for transcription in a host cell.

22 A vector as claimed in claim 20 or claim 21 which is suitable for expression of the nucleic acid in a mammal.

23 A cell containing heterologous nucleic acid of any one of claims 4 to 12, or the vector of any one of claims 20 to 22.

24 A cell transformed with heterologous nucleic acid of any one of claims 4 to 12, or the vector of any one of claims 20 to 22.

25 A cell as claimed in claim 23 or claim 24 which is selected from: a COS, CHO or HEK 293 cell or an oocyte.

26 A cell containing heterologous protein of any one of claims 1 to 3.

27 A method of influencing the electrophysiological and/or pharmacological properties of a cell, said method comprising the step of causing or allowing expression of heterologous nucleic acid as claimed in any one of claims 4 to 12 within the cell.

28 A method as claimed in claim 27 for increasing the VR-L ion channel activity in the cell.

29 A method as claimed in claim 27 for decreasing the VR-L ion channel activity in the cell.

30 A method as claimed in claim 29 comprising the use of any of (i) all or a distinctive part the nucleic acid of claim 12 such as to reduce VR-L activity by an anti-sense mechanism, (ii) a distinctive part of the nucleic acid of any one of claims 4 to 11 to reduce activity by co-suppression, or (iii) use of a ribozyme specific for a nucleic acid of any one of claims 4 to 11.

31 A transgenic non-human mammal, comprising a cell of any one of claims 23 to 26 and/or a cell in which the electrophysiological and/or pharmacological properties has been altered in accordance with the method of any one of claims 27 to 30.

32 A method for identifying a substance having ion-channel modulating activity, the method comprising the use of any of (i) a protein of any one of claims 1 to 3, (ii) a cell of any one of claims 23 to 26, (iii) a cell in which the electrophysiological and/or pharmacological properties has been altered in accordance with the method of any one of claims 27 to 30 (iv) the transgenic organism of claim 31.

33 A method as claimed in claim 32 comprising the steps of:
(i) exposing a heterologous protein of any one of claims 1 to 3, which is associated with a membrane or cell surface, to a solution of the substance such as to allow interaction between the substance and the protein,
(ii) measuring the electrophysiological response of the cell or membrane to this interaction.

34 A method as claimed in claim 32 or claim 33 for screening for analgesics or other compounds which affect

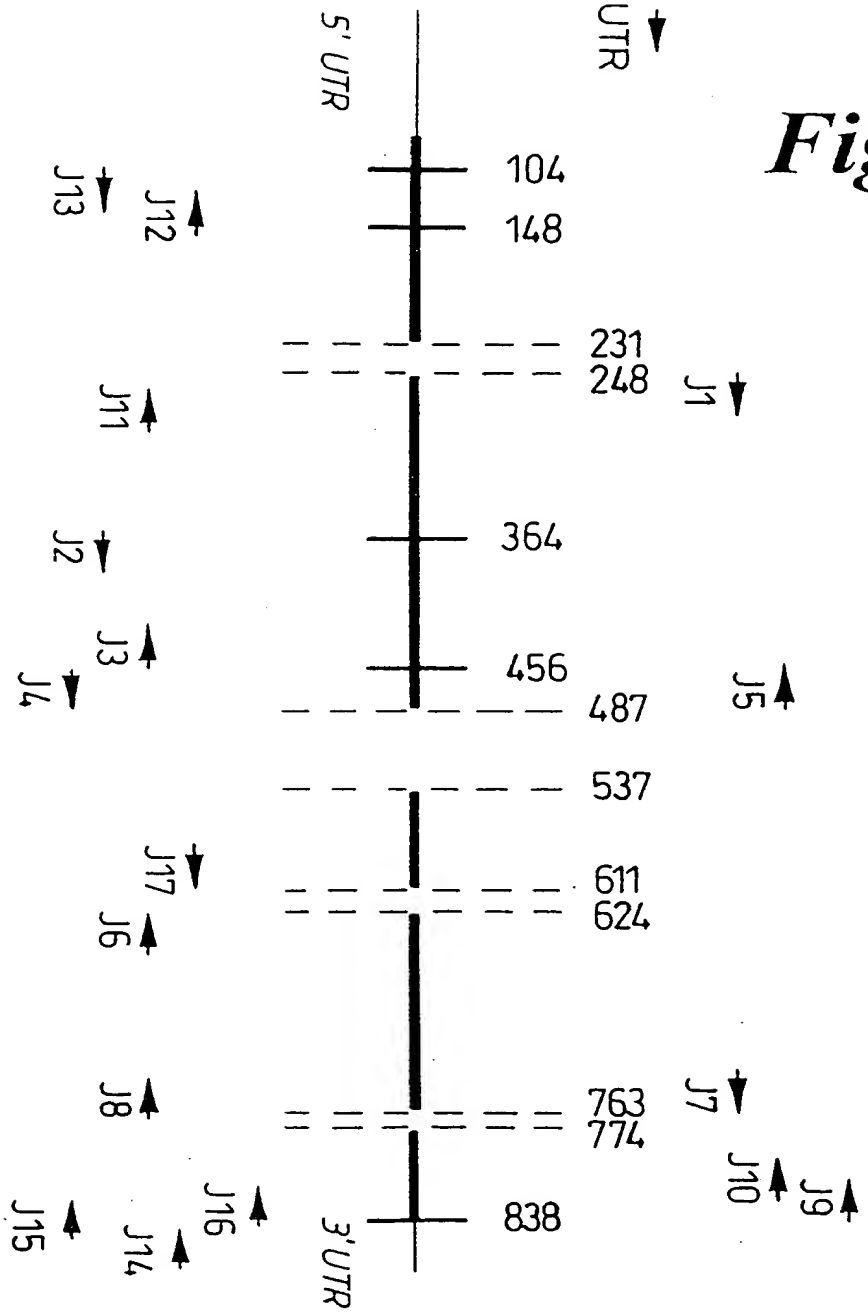
nociception; immunomodulatory agents; neuromodulatory agents.

35 A method of influencing the electrophysiological and/or pharmacological properties of a cell, said method comprising the step of modulating the activity of the protein of any one of claims 1 to 3.

36 A polypeptide comprising an antigen-binding site of an antibody capable of specifically binding the protein of claim 2.

37 Nucleic acid of any one of claims 4 to 12, or the vector of any one of claims 20 to 22, for use in gene therapy, or for use in the preparation of a medicament for use in gene therapy.

38 The nucleic acid or vector of claim 37 wherein the therapy is in respect of disorders associated with sensory neurons and/or cells of the immune system.


5' UTR
UTR
104
148
231
248
364
456
487
537
611
624
763
774
838
3' UTR
J1
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J3
J4
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J10
J11
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J16
J17

Fig. 1

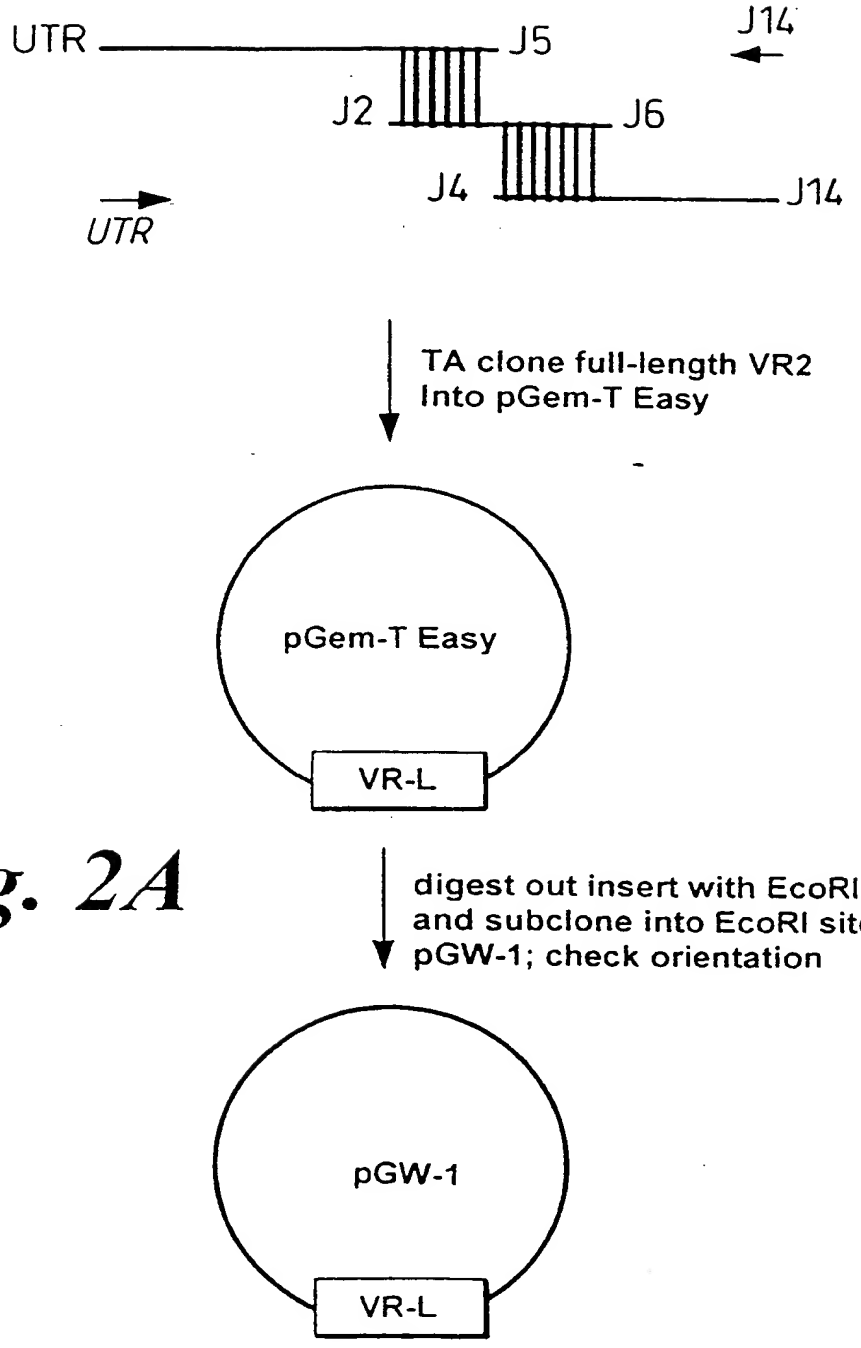
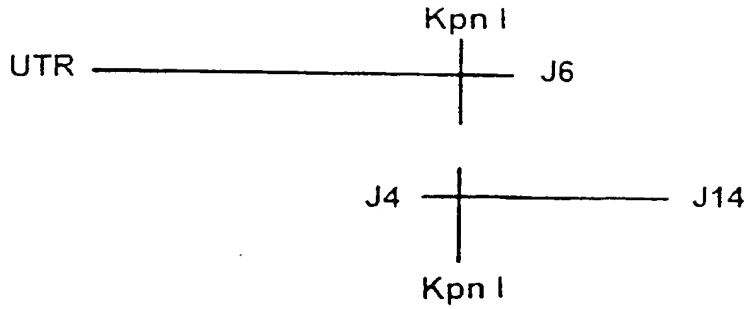

UTR
J5
J14
J2
J6
J4
J14
UTR
TA clone full-length VR2
Into pGem-T Easy
pGem-T Easy
VR-L
digest out insert with EcoRI
and subclone into EcoRI site of
pGW-1; check orientation
pGW-1
VR-L

Fig. 2A



* UTR/J6 was obtained by trans-splicing of 2 PCR products: UTR/J5 and J4/J6
* J4/J14 was obtained as a single PCR product

↓ clone individually into pGem-T Easy

Fig. 2B

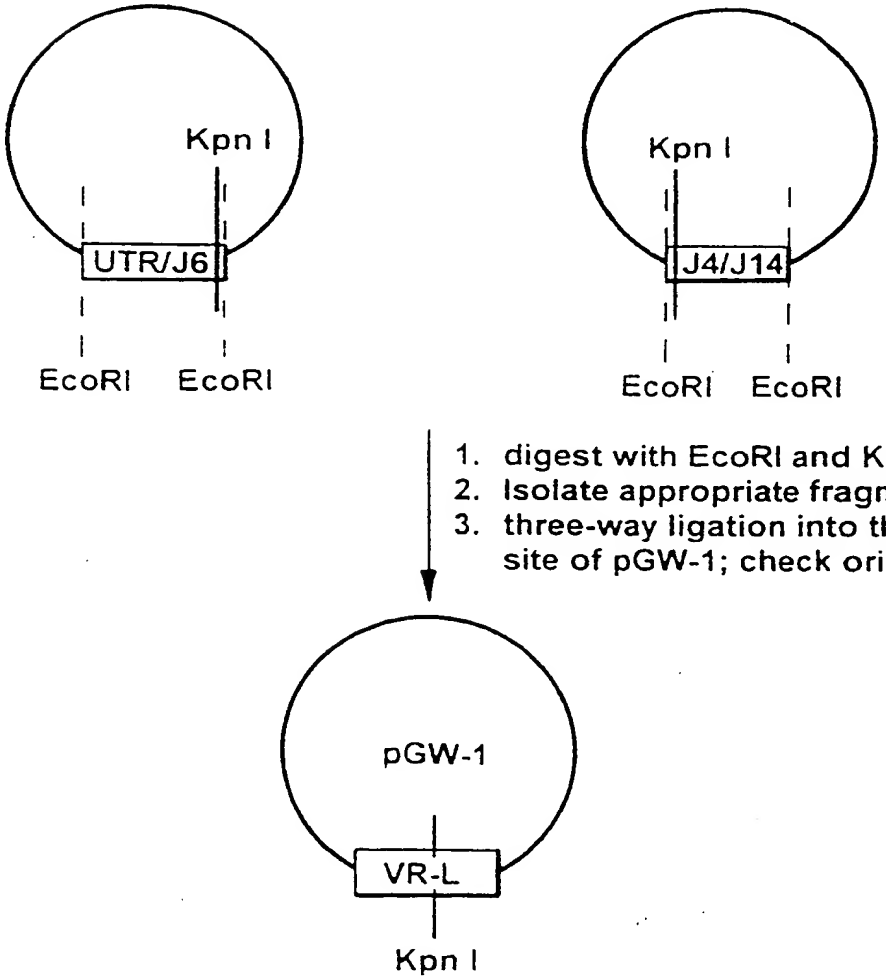
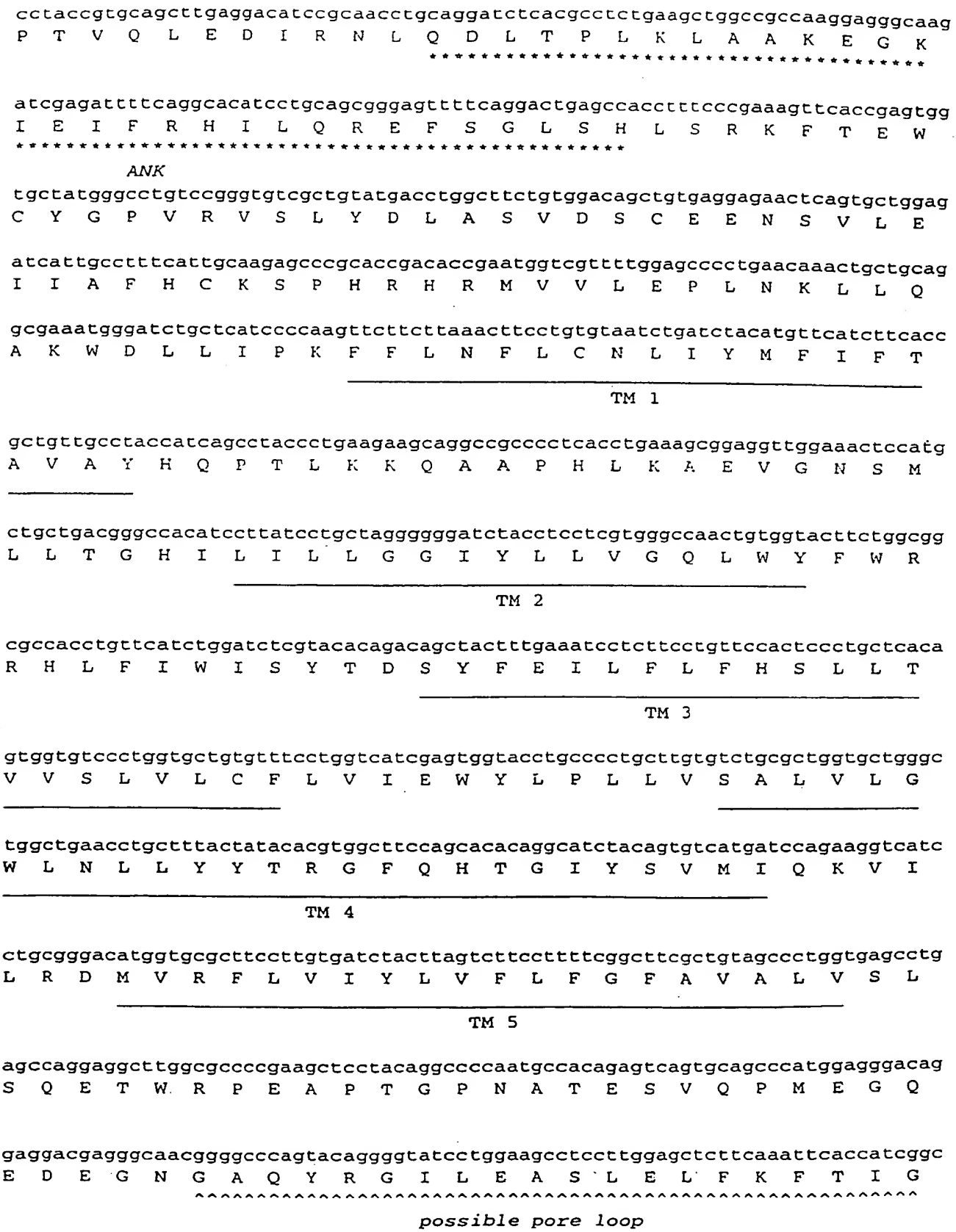


FIG 3A

Annotations: Ankyrin-like repeats (ANK), predicted transmembrane helices (TM) and the possible pore loop are designated with (*), (_) and (^), respectively.

```
ctagcctgtcctgacaggggagagttaagctcccgttctccaccgtgccggctggcaggtgggctgagggtgacc
gagagaccagaacctgcttgctggagcttagtgctcagagctggggagggaggttccgccgctcctctgctgtca
gcgccggcagcccctcccggcttcacttcctcccgcagcccctgctactgagaagctccgggatcccagcagccg
ccacgccctggcctcagcctgcggggctccagtcaggccaacaccgacgcgcagctgggaggaagacaggaccct

tgacatctccatctgcacagaggtcctggctggaccgagcagccccctcctcctaggatgacctcaccctccagc
                                                        M  T  S  P  S  S

tctccagttttcaggttggagacattagatggaggccaagaagatggctctgaggcggacagaggaaagctggat
S  P  V  F  R  L  E  T  L  D  G  G  Q  E  D  G  S  E  A  D  R  G  K  L  D

tttgggagcgggctgcctcccatggagtcacagttccagggcgaggaccggaaattcgcctctcagataagagtc
F  G  S  G  L  P  P  M  E  S  Q  F  Q  G  E  D  R_ K  F  A  S  Q  I  R  V

aacctcaactaccgaaagggaacaggtgccagtcagccggatccaaaccgatttgaccgagatcggctcttcaat
N  L  N  Y  R  K  G  T  G  A  S  Q  P  D  P  N  R  F  D  R  D  R  L  F  N

gtggtctcccggggtgtccccgaggatctggctggacttccagagtacctgagcaagaccagcaagtacctcacc
V  V  S  R  G  V  P  E  D  L  A  G  L  P  E  Y  L  S  K  T  S  K  Y  L  T

gactcggaatacacagagggctccacaggtaagacgtgcctgatgaaggctgtgctgaaccttaaggacggggtc
D  S  E  Y  T  E  G  S  T  G  K  T  C  L  M  K  A  V  L  N  L  K  D  G  V

aatgcctgcattctgccactgctgcagatcgaccgggactctggcaatcctcagcccctggtaaatgcccagtgc
N  A  C  I  L  P  L  L  Q  I  D  R  D  S  G  N  P  G  P  L  V  N  A  Q  C

acagatgactattaccgaggccacagcgctctgcacatcgccattgagaagaggagtctgcagtgtgtgaagctc
T  D  D  Y  Y  R  G  H  S  A  L  H  I  A  I  E  K  R  S  L  Q  C  V  K  L
                ************************************************************
                                                    ANK

ctggtggagaatggggccaatgtgcatgcccgggcctgcggccgcttcttccagaatggccaagggacttgcttt
L  V  E  N  G  A  N  V  H  A  R  A  C  G  R  F  F  Q  K  G  Q  G  T  C  F
**********************************

tattttggtgagctacccctctctttggccgcttgcaccaagcagtgggatgtggtaagctacctcctggagaac
Y  F  G  E  L  P  L  S  L  A  A  C  T  K  Q  W  D  V  V  S  Y  L  L  E  N
   ***************************************************************************
                                                    ANK

ccacaccagcccgccagcctgcaggccactgactcccagggcaacacagtcctgcatgccctagtgatgatctcg
P  H  Q  P  A  S  L  Q  A  T  D  S  Q  G  N  T  V  L  H  A  L  V  M  I  S
**************************

gacaactcagctgagaacattgcactggtgaccagcatgtatgatgggctcctccaagctggggcccgcctctgc
D  N  S  A  E  N  I  A  L  V  T  S  M  Y  D  G  L  L  Q  A  G  A  R  L  C
```

FIG 3A (cont.)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P T V Q L E D I R N L Q D L T P L K L A A K E G K
atcgagattttcaggcacatcctgcagcgggagttttcaggactgagccacctttcccgaaagttcaccgagtgg
I E I F R H I L Q R E F S G L S H L S R K F T E W
ANK
tgctatgggcctgtccgggtgtcgctgtatgacctggcttctgtggacagctgtgaggagaactcagtgctggag
C Y G P V R V S L Y D L A S V D S C E E N S V L E
atcattgcctttcattgcaagagcccgcaccgacaccgaatggtcgttttggagcccctgaacaaactgctgcag
I I A F H C K S P H R H R M V V L E P L N K L L Q
gcgaaatgggatctgctcatccccaagttcttcttaaacttcctgtgtaatctgatctacatgttcatcttcacc
A K W D L L I P K F F L N F L C N L I Y M F I F T
TM 1
gctgttgcctaccatcagcctaccctgaagaagcaggccgcccctcacctgaaagcggaggttggaaactccatg
A V A Y H Q P T L K K Q A A P H L K A E V G N S M
ctgctgacgggccacatccttatcctgctaggggggatctacctcctcgtgggccaactgtggtacttctggcgg
L L T G H I L I L L G G I Y L L V G Q L W Y F W R
TM 2
cgccacctgttcatctggatctcgtacacagacagctactttgaaatcctcttcctgttccactccctgctcaca
R H L F I W I S Y T D S Y F E I L F L F H S L L T
TM 3
gtggtgtccctggtgctgtgtttcctggtcatcgagtggtacctgcccctgcttgtgtctgcgctggtgctgggc
V V S L V L C F L V I E W Y L P L L V S A L V L G
tggctgaacctgctttactatacacgtggcttccagcacacaggcatctacagtgtcatgatccagaaggtcatc
W L N L L Y Y T R G F Q H T G I Y S V M I Q K V I
TM 4
ctgcgggacatggtgcgcttccttgtgatctacttagtcttccttttcggcttcgctgtagccctggtgagcctg
L R D M V R F L V I Y L V F L F G F A V A L V S L
TM 5
agccaggaggcttggcgccccgaagctcctacaggccccaatgccacagagtcagtgcagcccatggagggacag
S Q E T W R P E A P T G P N A T E S V Q P M E G Q
gaggacgagggcaacggggcccagtacaggggtatcctggaagcctccttggagctcttcaaattcaccatcggc
E D E G N G A Q Y R G I L E A S L E L F K F T I G
possible pore loop

FIG 3A (cont.)

```
atgggcgagctggccttccaggagcagctgcacttccgcggcatggtgctgctgctgctggcctacgtgctg
M  G  E  L  A  F  Q  E  Q  L  H  F  R  G  M  V  L  L  L  L  L  A  Y  V  L
^^^^
                                                                            ____

ctcacctacatcctgctgctcaacatgctcatcgccctcatgagcgagaccgtcaacagtgtcgccactgacagc
L  T  Y  I  L  L  L  N  M  L  I  A  L  M  S  E  T  V  N  S  V  A  T  D  S
_____________________________________________

   TM 6

tggagcatctggaagctgcagaaagcnatctntgtcctggagatggagaatggctattggtggtgcaggaagaag
W  S  I  W  K  L  Q  K  A  I  X  V  L  E  M  E  N  G  Y  W  W  C  R  K  K

cagcgggcaggtgtgatgctgaccgttggcactaagccagatggcagccccgatgagcgctggtgcttcagggtn
Q  R  A  G  V  M  L  T  V  G  T  K  P  D  G  S  P  D  E  R  W  C  F  R  V

gaggaggtgaactgggcttcatgggagcagacgctgcctacgctgtgtgaggacccgtcaggggcaggtgtccct
E  E  V  N  W  A  S  W  E  Q  T  L  P  T  L  C  E  D  P  S  G  A  G  V  P

cgaactctcgagaaccctgtcctggcttcccctcccaaggaggatgaggatggtgcctctgaggaaaactatgtg
R  T  L  E  N  P  V  L  A  S  P  P  K  E  D  E  D  G  A  S  E  E  N  Y  V

cccgtccagctcctccagtccaactgatggcccagatgcagcaggaggccagaggacagagcagaggatctttcc
P  V  Q  L  L  Q  S  N  Stp

aaccacatctgctggctctggggtcccagtgaattctggtggcaaatatatattttcactaactc
```

FIG 3B

```
  1 MEQRASLDSEESESPPQENSCLDPPDRDPNCKPPPVKPHIFTTRSRTRLFGKGDSEEASP
  1 ------MTSPSS-SPVFRLETLDGGQEDGSE----------ADRGK-LDFGSG----LPP

 61 LDCPYEEGGLASCPIITVSSVLTIQRPGDGPASVRPSSQDSVSAGEKPPRLYDRRSIFDA
 39 MESQFQGEDRKFAPQIRVN---LNYRKGTG--ASQP---D--------PNRFDRDRLFNA

121 VAQSNCQELESLLPFLQRSKKRLTDSEFKDPETGKTCLLKAMLNLHNGQNDTIALLLDVA
 83 VSRGVPEDLAGLPEYLSKTSKYLTDSEYTEGSTGKTCLMKAVLNLKDGVNACILPLLQID

181 RKTDSLKQFVNASYTDSYYKGQTALHIAIERRNMTLVTLLVENGADVQAAANGDFFKKTK
143 RDSGNPGPLVNAQCTDDYYRGHSALHIAIEKRSLQCVKLLVENGANVHARACGRFFQKGQ

241 GRPG-FYFGELPLSLAACTNQLAIVKFLLQNSWQPADISARDSVGNTVLHALVEVADNTV
203 GTC--FYFGELPLSLAACTKQWDVVSYLLENPHQPASLQATDSQGNTVLHALVMISDNSA

300 DNTKFVTSMYNEILILGAKLHPTLKLEEITNRKGLTPLALAASSGKIGVLAYILQREIHE
263 ENIALVTSMYDGLLQAGARLCPTVQLEDIRNLQDLTPLKLAAKEGKIEIFRHILQREFSG

360 PECRHLSRKFTEWAYGPVHSSLYDLSCIDTCEKNSVLEVIAYSSSETPNRHDMLLVEPLN
323 --LSHLSRKFTEWCYGPVRVSLYDLASVDSCEENSVLEIIAFH-CKSPHRHRMVVLEPLN

420 RLLQDKWDRFVKRIFYFNFFVYCLYMIIFTAAAYYRP-VEGLPPYKLKNTVGDYFRVTGE
380 KLLQAKWDLLIP-KFFLNFLCNLIYMFIFTAVAYHQPTLKKQAAPHLKAEVGNSMLLTGH

479 ILSVSGGVYFFFRGIQYFLQRRPSLKSLFVDSYSEILFFVQSLFMLVSVVLYFSQRKEYV
439 ILILLGGIYLLVGQLWYFWRRHLFIWISYTDSYFEILFLFHSLLTVVSLVLCFLVIEWYL

539 ASMVFSLAMGWTNMLYYTRGFQQMGIYAVMIEKMILRDLCRFMFVYLVFLFGFSTAVVTL
499 PLLVSALVLGWLNLLYYTRGFQHTGIYSVMIQKVILRDMVRFLVIYLVFLFGFAVALVSL

599 IEDGKNNSLPMESTPHKCRGSACK---PGN--SYNSLYSTCLELFKFTIGMGDLEFTENY
559 SQETWRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEASLELFKFTIGMGELAFQEQL

654 DFKAVFIILLLAYVILTYILLLNMLIALMGETVNKIAQESKNIWKLQRAITILDTEKSFL
619 HFRGMVLLLLLAYVLLTYILLLNMLIALMSETVNSVATDSWSIWKLQKAIXVLEMENGYW

714 KCMRKAFRSGKLLQVGFTPDGKDDYRWCFRVDEVNWTTWNTNVGIINEDPGNCEGVKRTL
679 WCRKKQ-RAGVMLTVGTKPDGSPDERWCFRVEEVNWASWEQTLPTLCEDP-SGAGVPRTL

774 SFSLRSGRVSGRNWKNFALVPLLRDASTRDRHATQQEEVQLKHYTGSLKPEDAEVFKDSM
737 EN----------------PVLASPPKEDEDGAS-EE--------NYVP--VQLLQSN-

834 VPGEK
    -----
```

FIG 4

```
RVR1     1 FSTAVVTLIEDGKNNSLPMES----TPHKCRGSACKP-GNSYNSLYSTCLELFKFTIGMG
MVR2     1 FAVALVSLSRDGRSPKAPEDSNTTVTEKPTLGQEEEP--VPYGGILDASLELFKFTIGMG
RVR2     1 FAVALVSLSREARSPKAPEDNNSTVTEQPTVGQEEEP--APYRSILDASLDLFKFTIGMG
JVR2     1 FAVALVSLSQETWRPEAPTGPNATESVQPMEGQEDEGNGAQYRGILEASLELFKFTIGMG

RVR1    56 DLEFTENYDFKAVFIILLLAYVILTYI
MVR2    59 ELAFQEQLPFRGVVLLLLLAYVLLTYV
RVR2    59 ELAFQEQLRFRGVVLLLLLAYVLLTYV
JVR2    61 ELAFQEQLHFRGMVLLLLLAYVLLTYI
```

Fig. 5

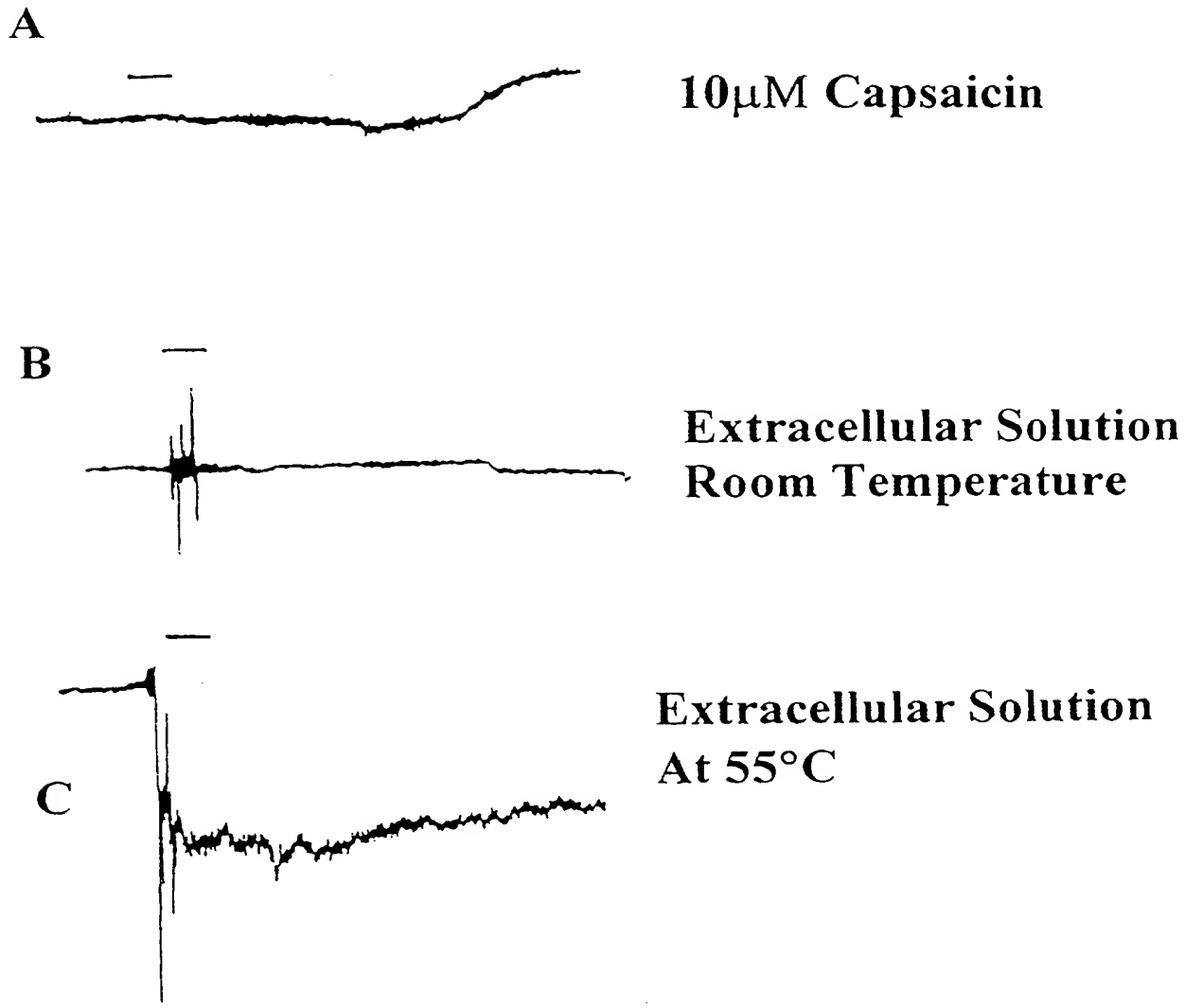
A
10μM Capsaicin
B
Extracellular Solution
Room Temperature
C
Extracellular Solution
At 55°C

Fig 6

Table 1. EST clones showing homology to rat vanilloid receptor subtype 1 (VR1).

GenBank Accession Number	Library/Tissue Source	Region of Homology to VR1 (amino acid nos.)
W44731	parathyroid tumour (H)	281-401
AA476107	mammary gland (M)	541-689
W38665	parathyroid tumour (H)	319-456
AA139413	kidney (M)	305-441
AA304033	aorta endothelial cells, TNF alpha-treated (H)	649-755
W82502	total fetus (mouse)	127-264
N23395	foreskin melanocytes (H)	248-401
AA281349	tonsillar cells enriched for germinal center B cells (H)	248-344
AA236416	Pooled melanocyte, fetal heart and pregnant uterus (H)	95-231
W92895	fetal liver and spleen (subtracted) (H)	537-658
AA741232	tonsillar cells enriched for germinal center B cells (H)	86-209
H20025	adult brain (H)	86-209
AA461295	total fetus (H)	86-209
AA357145	Jurkat T-cells (H)	364-456
AA321554	lymphoid tissue / CCRF-CEM cells, cyclohexamide treated (H)	774-838
H51393	adult brain (H)	87-209
AA768829	tonsillar cells enriched for germinal center B cells (H)	86-203
AA236417	Pooled melanocyte, fetal heart and pregnant uterus (H)	86-203

Fig 6(continued)

T12251	heart (H)	624-692
N26729	foreskin melanocytes (H)	119-209
H50364	adult brain (H)	127-209
W53556	embryos (M)	110-273
H21490	adult brain (H)	138-209
H49060	adult brain (H)	135-209
N21167	foreskin melanocytes (H)	127-209
N35179	foreskin melanocytes (H)	687-763
AA015295	placenta (M)	709-763
H27879	adult brain	127-203
AA281348	tonsillar cells enriched for germinal center B cells (H)	717-763
AA274980	total fetus (M)	722-763
AA078617	placenta (H)	325-389
N24224	foreskin melanocytes (H)	385-487
AA144832	testis (mouse)	200-354

Table 2. Primers Used for Cloning VRL fragments

Primer	Orientation	Accession. No. of EST clone (or THC) from where primer was designed	Closest analogous position in VR1 sequence (a.a. no.)	Primer Sequence (5' to 3')
J1	forward	W44731	248	GGT GAG CTA CCC CTC TCT TTG
J2	forward	AA357145	364	CAC CTT TCC CGA AAG TTC ACC
J3	reverse	N24224	456	AGG CTG ATG GTA GGC AAC AGC
J4	forward	N24224	487	CCA CAT CCT TAT CCT GCT AG
J5	reverse	N24224	487	CTA GCA GGA TAA GGA TGT GG
J6	reverse	W92895	624	CAA GGA GGC TTC CAG GAT ACC
J7	forward	AA281348	763	GAG GTG AAC TGG GCT TCA TGG
J8	reverse	AA281348	763	CCA TGA AGC CCA GTT CAC CTC
J9	reverse	AA321554	838	TCA CTT CTC CCC GGA AGC GGC AG
J10	reverse	AA321554	838	GAA GAC CTC AGC GTC CTC TGG
J11	reverse	W44731	248	CAC ATC CCA CTG CTT GGT GCA
J12	reverse	H21490	148	TGT GTA TTC CGA GTC GGT GAG
J13	forward	H20025	104	ACC GAG ATC GGC TCT TCA ATG
J14	reverse	(THC161190)	3' UTR (47 bp from stop)	GAA AGA TCC TCT GCT CTG TCC
J15	reverse	(THC161190)	3' UTR (30 bp from stop)	GTC CTC TGG CCT CCT GCT GCA
J16	reverse	(THC161190)	stop codon	TCA GTT GGA CTG GAG GAG CTG
J17	forward	W92895	606	GAA GCT CCT ACA GGC CCC AAT G
UTR	forward	(THC176254)	5' UTR (165 bp 5' of initiation Met)	TGC TAC TGA GAA GCT CCG GGA TCC

Fig. 7